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ORIGINAL ARTICLE

Evaluation and Genetic Polymorphism studies of Jatropha (*Jatropha curcus*) for Water Stress Tolerance

Borse Tushar*, Kulkarni Manoj and Chaphalkar Sushama

Vidya Pratishthan's School of Biotechnology Vidyanagari, Bhigwan Road, Baramati 413133 INDIA.

Phone: 91-2112-239388; 91-2112-239386

*Email-thbvsbt@gmail.com

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Jatropha (*Jatropha curcus*) is an alternative resource for biodiesel. To boost the rural economy in sustainable manner it is estimated that 30 Million hector plantation may replace current use of fossil fuel. Although Jatropha has an inbuilt ability to grow under water limited conditions, scanty information is available about natural genetic variation for water stress tolerance. Three local genotypes from Pune district were collected and initially screened by imparting artificial stress using PEG – 6000. Seedlings were subjected to increasing concentration of PEG – 6000 (30, 60, 90, 120 and 150 gm/l) to study effect on growth parameters. The root growth, number of secondary roots, true leaf expansion at morphological level and palisade mesophyll height, xylem vessel expansion at anatomical level showed drastic negative impact as compared to control. It is worth to note that local germplasm performance was categorized into susceptible group as compared to tolerant genotype [Chattisgadh Selection] indicating need for genetic improvement. These genotypes were further studied at molecular level with RAPD and ISSR markers to amplify genetic variation. Polymorphic bands from Chattisgadh selection genotype are being evaluated for their usefulness as markers for water stress tolerance.

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Jatropha curcas L. (purging nut, physic nut, barbadose nut or sabudum), a member in the family Euphorbiaceae, is a large shrub with multipurpose uses. To boost the rural economy in sustainable manner it is estimated that 30 Million hector plantation may replace current use of fossil fuel.

There is a growing interest in *Jatropha curcas* as a biodiesel plant to help alleviate the energy crisis Jatropha is becoming a poster child among some proponents of renewable energy and appropriate technology, especially as an oil-bearing, “drought resistant” tree for marginal lands for small farmers. Not to be forgotten, marginal yields are obtained from plants grown on marginal lands. To be economical as a biodiesel fuel, Jatropha must be produced in volume. Physic nut has been the important target in many countries for substitute energy. Seed kernel contains about 33-60% oil (Gübitz et al., 1997). Various parts of the plant have been used for many aspects (Heller, 1996). In the present scenario, the need of plants is increasing from government institutes, private sectors and farmers for research; development and producing stem cuttings, seeds, oil and substitute energy.

In the present study we have tried to evaluate the locally available Jatropha germplasm to identify promising cultivars for elevated drought tolerance. Initially three genotypes from Pune district were colleted and compared to with genotype from Chattisgadh. All four genotypes were initially screened in field conditions for water stress tolerance, two genotypes ‘Purandar Selection’ being adopted to local conditions and new introduction ‘Chattisgadh Selection’ being stay green habit were selected for *in vivo* and genetic polymorphism studies.

For *in vitro* and *in vivo* studies in relation to drought tolerance, most widely used solute is the polymer, Polyethylene Glycol (PEG). PEG compounds have been used with monocots, dicots, gymnosperms, fungi and yeasts to induce water stress (Nepomuceno *et al*., 1998). Decrease in seedling growth as a result of the decrease in osmotic potentials with increasing PEG concentration has been reported in Tomato (Kulkarni and Deshpande, 2006), Rice (Pirdashti *et al*., 2003), Wheat (Dhanda *et al.,* 2004), and Cowpea (Badiane *et al*., 2004). Kulkarni and Deshpande (2006) reported 10 mg average root dry weight in drought tolerant mutant as compared to 6.5 mg average root dry weight in susceptible cultivated genotypes of tomato. Polyethylene Glycol (6000 M.W.) with increasing concentration from top (0, 30, 60, 90, 120 and 150 g/l) represents increasing stress levels. Stability of susceptible and resistant genotype for morphological and anatomical features was studied with an objective to discriminate seedling growth in Jatropha genotypes under water stress conditions.

Kulkarni and Deshpande (2006) reported RAPD based DNA polymorphism in mutant and cultivated tomato genotypes differentiated initially based on anatomical differences and PEG- 6000 based screening. Shashidhar *et al*., (2000) identified RAPD markers for maximum root length in rice. These markers were co-dominant in nature. Marker aided selection strategies are discussed with reference to develop drought tolerant cultivars in Rice and Pearl Millet (Hash *et al.,* 2000 a, b). The accomplished and proposed strategies in Maize (Veldboom and Lee, 1996; Ribaut and Betran, 1999), Pearl Millet (Yadav *et al.,* 1999, Hash and Barmel-cox, 2000) were discussed.

Distinct genotypes were subjected to DNA polymorphism studies using RAPD and ISSR primers. Polymorphic DNA bands were amplified and will further be used for cloning and characterization to asses their utilization as drought tolerance markers in *Jatropha curcus*. Jatropha is inherent drought resistant plant, but it was interesting to know that substantial genetic variation is present in Jatropha genotypes and needs to be further explored.

MATERIALS AND METHODS

Seedling *in Vivo* culture: The seeds were surface sterilized with 70 % ethanol for 1 minute and then with mercuric chloride (0.1 %) for 10 minutes and thoroughly washed with sterile distilled water for four times. The seeds were presoaked with sterile water for one day and the next day sown onto autoclaved cocopit in test tubes. For *in vivo* screening, Jatropha genotypes were grown in test tubes using 2 gm autoclaved cocopeat with adding 3 ml different concentrations of PEG - 6000 @ 0, 30, 60, 90, 120 and 150 g/l solutions alternate days. The tubes were maintained under optimum growth conditions at 16 hrs photoperiod (70-µ mol. M^2 Sec^{-1}) and 28^0C temperature. Seedling growth was recorded 25 days after sowing. Root and shoot length (cm) as well as their respective fresh and dry weight (mg) were recorded for *in vivo* grown seedlings.

DNA polymorphism studies:

DNA extraction and quantification: Total genomic DNA was extracted using the CTAB DNA extraction protocol described by Kulkarni and Deshpande (2006) with some modifications. Higher concentration of PVP (2 %) and β - mercaptoethanol (2%) was used to overcome high concentration of polyphenols and other secondary metabolites. The DNA was further purified using ion exchange chromatography, quantity and quality of DNA was assessed by electrophoresis in 0.8 % agarose gel against 100 ng λ DNA as standard. DNA was diluted to final concentration of 50 ng/µl for PCR amplification.

PCR amplification: RAPD-PCR was performed in a reaction volume of 25 µl containing 2.5 mM each of dNTPs, 2.5 µl of 10 X PCR assay buffer, 2 µl 0f 25 mM $MgCl_2$, 2 µl of 10 µM oligonucleotide primers (Integrated DNA Technologies, Inc. USA), 100 ng of genomic DNA template and Taq DNA polymerase. Amplifications were performed in PTC 200 "DNA Engine" cycler (Pletier thermal cycler, Germany).

Total 50 random primers were selected for RAPD-PCR analysis. PCR amplification was carried out with initial denaturation at 94^0C for 4 minutes followed by the amplification programme of 35 cycles of denaturation – 94^0C for 20 seconds, annealing according to Tm of each primer for 30 seconds and extension at 72^0C for 1 minute. The 35^{th} cycle was followed by final extension step at 72^0C for 4 minutes.

ISSR-PCR reaction mixture involved 2.5 mM each of dNTPs, 2.5 µl of 10 X PCR assay buffer, 2.5 µl 0f 25 mM $MgCl_2$, 2.5 µl of 10 µM oligonucleotide primers (Operon technologies, Inc. USA), 100 ng of genomic DNA template and 1 unit of Taq DNA polymerase. Total 20 ISSR primers were used for amplification. PCR amplification was carried out using programme having Initial denaturation at 94^0 C for 7 minutes followed by the amplification programme of 40 cycles of denaturation – 94^0C for 1 minute, annealing according to Tm of each primer for 1 minute and extension at 72^0C for 2 minutes. The 40^{th} cycle was followed by final extension step at 72^0C for 10 minutes. PCR products were run on 1.5 % agarose gel with 5V/cm current and stained with ethidium bromide. λ DNA Hind III / Eco RI double digest and super mix DNA ladder were used as molecular weight marker.

RESULTS AND DISCUSSION

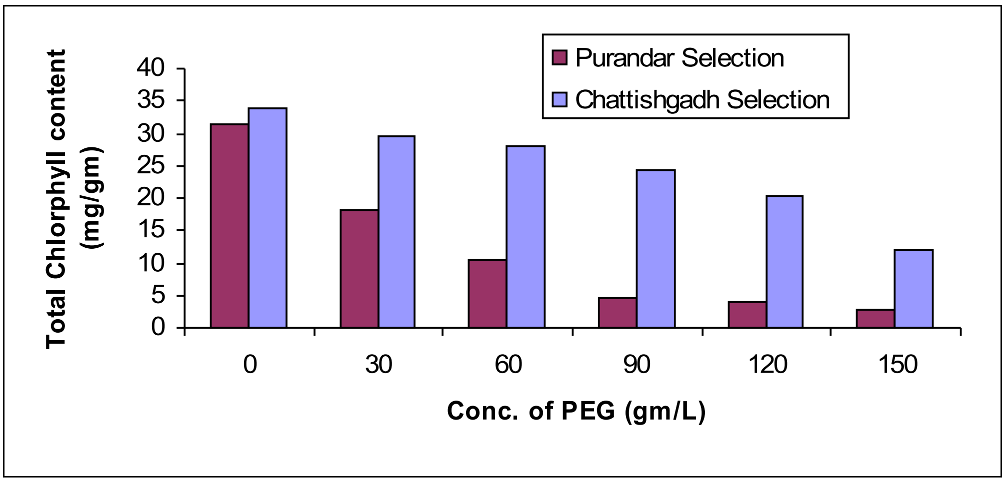
Field screening of three local varieties from Pune district i.e. Purandar Selection, Baramati Selection and Baburdi Selection was done in comparison to new introduction Chattisgadh selection. Varieties Purandar selection and Chattisgadh selection were selected based on their discrimination towards stay green characters under field stress. These results were further confirmed by *in vivo* studies under different levels of stress. Under PEG- 6000 artificial stress situation, growth of both genotypes was retarded with respect to root, shoot growth and stay green

Table1: Morphological variation in two discriminative Jatropha genotypes under water stress conditions.

Characters	Varieties/Conc. of PEG (gm/L)	0	30	60	90	120	150
Leaf fresh weight (mg)	Purander selection	1330	300	296	272	45	37
	Chattishgadh selection	1525	1121	1060	896	812	496
Stem fresh weight (mg)	Purander selection	2070	1290	1460	1022	642	568
	Chattishgadh selection	2870	2320	1379	1295	1161	1025
Root length (cm)	Purander selection	5	2	1.5	2.5	3.5	1.8
	Chattishgadh selection	4	6.5	6.9	7.6	6.5	6.2
No of secondary roots	Purander selection	47	10	16	20	36	13
	Chattishgadh selection	37	70	74	62	52	32

Table 2: Anatomical variation in two discriminative Jatropha genotypes under water stress conditions

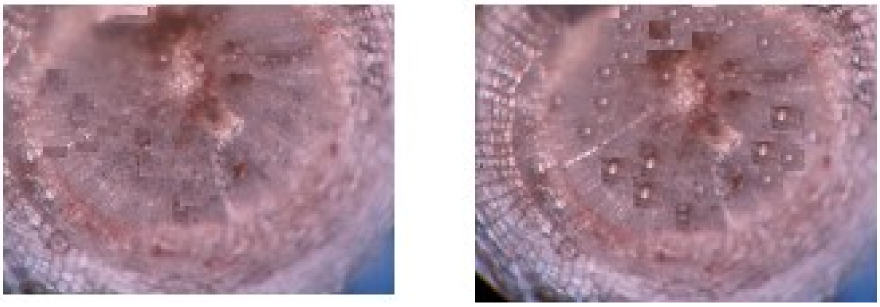
Characters	Varieties/Conc. of PEG (gm/L)	0	30	60	90	120	150
No of stomata	Purander selection	235	313	347	291	222	170
	Chattishgadh selection	112	128	118	102	90	85
PM: SM ration	Purander selection	0.428	0.307	0.266	0.250	0.0.321	0.178
	Chattishgadh selection	0.636	0.428	0.456	0.532	0.502	0.333
No of xylems in roots	Purander selection	30	21	17	15	10	7
	Chattishgadh selection	42	35	34	28	19	18
Diameter of xylem in roots (µm)	Purander selection	28	21	16	15	14	12
	Chattishgadh selection	42	35	32	28	26	21



Graph 1: Compairing chlorophyll stability under water stress situation

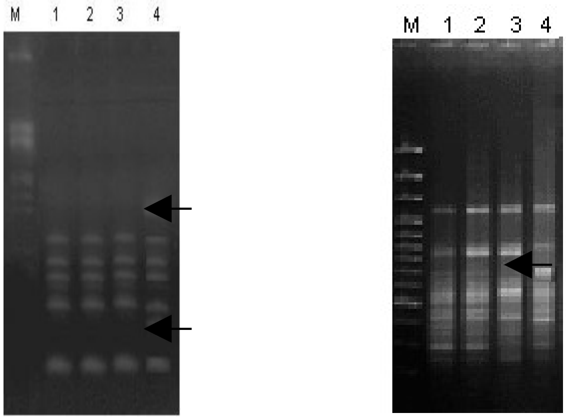
Chattisgadh selection **Purandar selection**

Fig 1. Comparative seedling growth at 120 gm/l concentration



Purandar Selection **Chattisgadh Selection**

Fig 2. Difference in number of xylem vessels demonstrated in root cross sections



(a) **(b)**

Fig 3. Marker amplification for understanding genetic variation

(a) **RAPD polymorphism with primer 5' TTAACCCGGC 3';**

Lane M : λ DNA Eco RI/ Hind III double digest, 1- Baburdi Selection, 2 - Baramati selection, Purandar Selection and 4 – Chattisgadh Selection

(b) **ISSR polymorphism using primer 5' $(CA)_8$ G 3'**

Lane M: Supermix DNA ladder, 1- Baburdi Selection, 2 - Baramati Selection, Purandar Selection and 4 – Chattisgadh Selection character.

It is clear from Table 1 that, leaf and stem fresh weight decreased by 97 and 73 % for Purandar Selection and 77 and 65 % for Chattisgadh Selection respectively at highest concentration of 150 gm/l. These results indicate higher ability of biomass production by Chattisgadh Selection as compared to Purandar Selection under stress. The root growth was promoted by water stress situation, as stress increased root length was observed to increase. These results are in accordance with Kulkarni and Deshpande (2006). Number of feeder roots maintained as secondary roots under stress situation are important character exhibited by stress tolerant genotype as observed in Chattisgadh Selection. Similar results studying PEG mediated stress tolerance studies are reported by Rice Pirdashti *et al*., 2003, Dhanda *et al.,* 2004, and Badiane *et al*., 2004 in agricultural crops.

Recent reports emphasize anatomical features playing important role in water stress tolerance in plants. Whole plant anatomical approach was considered to understand effect of water stress on anatomical features in Jatropha. Stress tolerant genotype Chattisgadh selection had only 45 – 50 % stomata on lower side of leaf as compared to stress susceptible local cultivar Purandar Selection. Reduced number of stomata maintains better evapotranspiration rate and turgor pressure in leaves. Lower number of stomata associated with water stress tolerance are earlier reported (Kulkarni et al., 2007) in grape (*Vitis venifera*). Better Tissue ratio (Palisade Mesophyll: Spongy Mesophyll) was maintained by genotype Chattisgadh Selection as compared to rapidly lowering tissue ratio in Purandar Selection genotype with increasing water stress. Higher magnitude of number of xylem vessels and maintaining xylem diameter with increasing stress is essential feature for water stress tolerance. Purandar selection genotype was unable to maintain xylem vessel growth and diameter with increasing water stress can be considered as main reason for susceptibility to drought condition [Fig 1 and 2]. Stability of total chlorophyll content is an important parameter to discriminate genotypes for drought tolerance. Graph 1 clearly indicates ability of genotype Chattisgadh Selection to maintain higher total chlorphyll content even at higher stress conditions. Comparatively second genotype under study shows drastically reducing chlorophyll level with increasing water stress, being main reason for lower magnitude of biomass production as evidenced in Table 2.

Based on artificial water stress tolerance studies, genetic variation was amplified using 50 RAPD and 20 ISSR primers alnogwith two additional local varieties. Polymorphic bands were observed by different primers. Two Polymorphic bands in variety Chattisgadh Selection by primer 5' TTAACCCGGC 3' with appox. 1.95 Kb and 900 bp molecular weight were amplified. ISSR primer 5' $(CA)_8$ G 3' amplified polymorphic marker; result is shown in Fig 3 (b) with appox. 1.5 kb polymorphic product amplified. Selected amplifications from these dominant (RAPD) and co-dominant (ISSR) markers will be cloned and further characterized based on sequence information to develop functional Sequence Tagged Site (STS) markers for drought tolerance.

Present investigation is an attempt to characterize locally adapted germplasm in Jatropha curcus to select suitable genotypes, which could be used for further genetic enhancement in relation to better drought tolerance. Jatropha being a major crop of barren lands, it's cultivation always had threat of lower yields due to stress during flowering and fruiting period. Identification of drought resistant sources like Chattisgadh selection and utilization of molecular markers amplified will help in identifying sound genetic base as donor genotype for drought tolerance studies in *Jatropha curcus*. Finer studies and introgression of these markers will help in near future to attain goal of "Yield Under Stress" in Jatropha.

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